In vivo gene transfer into rat arterial walls with novel adeno-associated virus vectors

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Purpose: **We studied the ability of recombinant adeno-associated virus (rAAV) vectors to achieve gene transfer in vivo to intact rat carotid arteries.**
Methods: **Isolated segments of uninjured rat carotid arteries were incubated with (1) rAAV vectors that expressed a β-galactosidase gene, (2) a related vector with no promoter, or (3) a normal saline solution. Gene transfer was evaluated with in situ polymerase chain reaction (PCR). Transgene expression was assessed at intervals that ranged from 24 hours to 2 months by measurement of β-galactosidase activity and protein mass in tissue extracts with fluorometric and enzyme-linked immunosorbent assays, respectively. Dose dependence of expression was determined for virus concentrations that ranged from 5×10^4 to 5×10^5 infectious units (iu)/ml.**
Results: **Light microscopic analysis of in situ PCR-stained histologic sections of transduced vessel walls showed approximately 90% of intimal and medial cell nuclei contained the β-galactosidase gene, compared with none in control arteries. In vivo β-galactosidase expression was (1) highest 24 hours after gene transfer, (2) elevated for 1 month, and (3) dose responsive.**
Conclusions: **rAAV vectors can mediate focal gene transfer into the intact rat carotid artery with detectable levels of transgene expression for 1 month and are potentially useful agents for in vivo gene transfer into intact arteries. (J Vasc Surg 1997;25:347-55.)**

The feasibility of gene transfer as an approach to treat vascular disease has been established. Gene therapy strategies in living animals for inhibiting arterial intimal hyperplasia,[1,2] delaying vein graft atherosclerosis,[3,4] and preventing thrombosis,[5,6] as well as for promoting angiogenesis in humans,[7] have recently been reported. However, the fundamental issue of selecting the best agent for arterial gene delivery remains unresolved. The vehicles currently used are either viral or nonviral. Infectious recombinant viral vectors, because of natural host-virus affinity, may be the most efficient agents.[8]

Adeno-associated virus (AAV) is a mammalian transduction vector with characteristics that make it promising as an agent for human gene therapy, including (1) the ability to infect a broad range of human cell types in culture with high efficiency and to latently infect nondividing cells with stable and efficient integration of viral DNA,[8,9] (2) the tendency for site-specific integration into a distinct 7-Kb region of human chromosome 19,[10,11] (3) the expression of a minimal number of viral antigens to induce a host immune response,[9] and (4) the lack of associated symptoms or disease subsequent to human infection, although 80% of adults are seropositive for antibodies against AAV.[9]

Recombinant AAV (rAAV) vectors have potential advantages over retroviral vectors, including physical stability, which allows concentration to titers of 10^{10},[12] and the ability to mediate gene transfer and expression in vitro into nondividing cells.[13,14] Also, in contrast to adenoviral vectors, rAAV vectors lack cytopathogenicity.[15-17]

Because of the potential advantages of rAAV vectors, we initiated studies to determine the feasibility of in vivo arterial gene transfer with these agents. Previous work in our laboratory showed tropism of wild-type AAV (wtAAV) for cultured primary human umbilical vein endothelial cells (HUVECs), as well as for rat arterial intimal and smooth muscle cells in

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vivo. In addition, we used rAAV vectors encoding the β-galactosidase gene (lacZ) to transduce HUVECs in vitro, with β-galactosidase expression detectable by histochemical staining with XGAL in 5% to 8% of cells (unpublished data from Dmitri Gnatenko, PhD, SUNY at Stony Brook).

The focus of this study was to establish the ability of rAAV vectors that contained lacZ to transduce arterial wall cells by temporary direct exposure in vivo in an intact rat carotid artery model. The purpose of the study was to determine (1) the feasibility of in vivo arterial rAAV gene transfer, (2) the level and duration of recombinant gene expression consequent to gene transfer, and (3) the dose dependence of transgene expression.

MATERIAL AND METHODS

Construction of rAAV vectors. Three plasmids were used to construct rAAV stocks for this study. The plasmid pTRColα(I)βgeo (provided by Dr. A. D. Miller, Seattle, Wash.) contained the murine collagen α(I) promoter upstream to the β-galactosidase-NEO fusion gene (βgeo) and the polyadenylation signal of SV40 early genes (pA_E), flanked by AAV inverted terminal repeats (TRs). Plasmids pTRCMVβ and pTRβ were generated by standard techniques with use of the backbone plasmid pBR322, as previously described.[18,19] pTRCMVβ contained the cytomegalovirus (CMV) immediate-early promoter and enhancer upstream to the late gene 16S/19S splice donor/splice acceptor signal of SV40 (SD/SA), upstream to lacZ, the polyadenylation signal of SV40 late genes (pA_I), and pA_E in order, flanked by TRs. pTRβ contained SD/SA upstream to lacZ, pA_I, and pA_E in order, flanked by TRs, and thus contained no promoter.

rAAV viral stocks were prepared and titered by use of established procedures.[19-21] Human 293 (adenovirus type-5 transformed embryonic kidney) cells were plated at a density of 5×10^6 cells/100 mm^2 dish, and 5 μg of individual cesium-banded plasmids [pTRColα(I)βgeo, pTRCMVβ, or pTRβ] were used to transfect 293 cells with use of the calcium phosphate precipitation method of Chen and Okayama.[20] Cotransfections were completed at 39.5° C in 3% carbon dioxide with 15 μg of the complimenting plasmid pIM45—which is defective for packaging but supplies the wtAAV gene products *in trans*—and 2×10^7 plaque-forming units (pfu) of adenovirus ts149 (a temperature-sensitive strain that is nonpermissive at more than 39° C). Approximately 48 hours after initial transfections, lysates were prepared by freezing and thawing, contaminating adenovirus was inactivated by 55° C incubation for 60 minutes, and cellular debris was pelleted by centrifugation at 2000 rpm for 10 minutes.

rAAV titers determined by replication center assay, as described previously.[22] In brief, in microdilution plates, approximately 2.5×10^4 293 cells were infected with rAAV stocks constructed with the rAAV plasmids pTRColα(I)βgeo, pTRCMVβ, or pTRβ. Serial dilutions of the rAAV, adenovirus [multiplicity of infection (MOI), 20 pfu/cell] and wtAAV (MOI 2), were added to each well. A control well was infected with adenovirus alone (no rAAV or wtAAV), and adenovirus contamination was excluded by replication center assays in the absence of exogenous adenovirus. After 30 hours, cells were removed using trypsin, and they were resuspended and trapped on nitrocellulose filters under suction. Filters were then hybridized to β-galactosidase gene (lacZ) [^{32}P]DNA to detect the recombinant gene. The total number of dots on autoradiographs of each filter disk was estimated to determine the relative titers of individual rAAV stocks (data not shown). Titers were calculated as described by Hermonat and Muzyczka[19] and expressed as infectious units per milliliter (iu/ml).

Stocks were evaluated for evidence of contaminating wtAAV in experiments completed in parallel with titering. One microliter of the rAAV and adenovirus (MOI, 20 pfu/cell) was added to each well of 293 cells, as described for titering. Filters were similarly prepared but were then hybridized to wild-type [^{32}P]DNA to detect possible contamination. No evidence of wtAAV contamination existed on autoradiographs of the filter disks, with an estimated sensitivity of 100 iu/ml (data not shown).

Stocks generated included (1) rAAV/Col, driven by the murine collagen α(I) promoter; titer = 5.0×10^5 iu/ml, (2) rAAV/CMV, driven by the CMV immediate-early promoter and enhancer; titer = 4.5×10^5 iu/ml, and (3) rAAV/pTRβ, the control vector with no promoter; titer = 3.0×10^5 iu/ml. Av1LacZ4, a recombinant replication-deficient adenovirus encoding lacZ, was constructed as described previously[23] and used as a positive control, titer = 3×10^{10} pfu/ml; (provided by Dr. B. Trapnell, Genetic Therapy Inc., Gaithersburg, Md.).

To select the vector with the highest level of expression, HUVECs were transfected in vitro with individual rAAV constructs, and β-galactosidase expression in these cells was evaluated with quantitative β-galactosidase activity 4-methylumbelliferyl-β-galactoside (MUG) assays. rAAV/Col and rAAV/CMV showed protein expression, with β-galactosidase activity approximately 400% and 200% above control,

respectively (unpublished data from Dmitri Gnatenko, PhD, SUNY at Stony Brook, April 1995). These observations supported the decision to proceed with in vivo studies, with use of rAAV/Col primarily, and to study rAAV/CMV at the 24-hour interval only.

Animals. All animal procedures complied with the "Principles of Laboratory Animal Care" (from the National Society for Medical Research) and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 86-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee of the SUNY at Stony Brook. Adult male Sprague-Dawley rats (Taconic Farms, Inc., Germantown, N.Y.) that weighed 450 to 650 gm were used. Rats were anesthetized with intramuscular injections of ketamine (83 mg/kg, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and xylazine (8.3 mg/kg, Miles Inc., Shawnee Mission, Kan.). After surgery, rats were returned to their cages and allowed water ad libitum.

In vivo gene delivery. The right carotid artery was surgically exposed, and a segment of common carotid artery ≈1 cm long was isolated between vascular clamps. An arteriotomy was performed in the external carotid artery. A 24-gauge plastic catheter was introduced through the arteriotomy, and the isolated vessel segment was flushed with normal saline solution before instillation of 50 μl of either vector or control solution. Intraluminal pressures, measured with a standard pressure transducer during several vector infusions under usual conditions, ranged from 150 to 190 mm Hg (data not shown). rAAV stocks were thawed and maintained on wet ice until use. All stocks were used within 2 hours of thawing. After 30 minutes of incubation, the instilled solution was withdrawn, the external carotid artery was ligated, and blood flow through the common and internal carotid arteries was reestablished.

At varying time intervals after gene transfer, animals were reanesthetized, and the right common carotid artery was harvested. In addition, the left carotid artery was dissected from each animal and processed in the same manner as the right artery to serve as a paired control. The anesthetized animal was then killed by ether inhalation. At the time the animal was killed, all common carotid arteries were patent.

Detection of transgene (LacZ) delivery with in situ DNA polymerase chain reaction (PCR). Artery segments for DNA in situ PCR were removed, washed in phosphate buffered saline solution, fixed in 10% neutral buffered formalin, and then frozen in OCT (Miles, Inc, Elkhart, N.Y.) by immersion in liquid nitrogen. Cryosections of 4 μm were then placed on silane-coated slides and evaluated for viral incorporation with β-galactosidase–specific oligonucleotide primers (5′-AAG CAG CGT TGT TGC AGT GCA-3′ [base pairs (bp) 2456-2476]; 5′-ATA TTC AGC CAT GTG CCT TCT-3′ [bp 2957-2937]), and DNA in situ PCR.[24] In brief, sections were digested with pepsin (2 mg/ml) for 30 minutes at 25° C and washed in diethyl pyrocarbonate–treated water. Products were then amplified with digoxigenin-11-dUTP and "hot-start" PCR to inhibit nonspecific mispriming. Segments from paired left arteries were used as negative controls. For positive controls, arterial segments were evaluated with primers specific for rat β-actin (5′-CAG GCT GTG TTG TCC CTG TAT-3′ [bp 913-934]; 5′-TTC ATG GAT GCC ACA GGA TTC-3′ [bp 1399-1420]). Slides were then washed in xylene for 5 minutes, rinsed in 100% ethanol, and vacuum-dried before immunodetection with the alkaline phosphatase–conjugated anti-digoxigenin antibody (diluted 1:200 at 25° C for 30 minutes). Detection was completed with the substrates nitroblue tetrazolium and BCIP for 10 minutes. Sections were counterstained with nuclear fast red and examined by light microscopy for the presence of blue-black nuclear amplification and staining, indicative of lacZ transfer. Representative sections of arterial segments were stained with hematoxylin and eosin to evaluate for the presence of intimal and medial cell necrosis and acute inflammatory cell infiltration.

Estimation of transduction frequency. To estimate the transduction frequency in the vessel wall, three PCR-stained histologic sections from each of two arteries (n = 6) transduced with rAAV/Col or rAAV/CMV were analyzed with light microscopy. An estimate of the number of cell nuclei that showed lacZ transfer per section was obtained, as well as the total number of nuclei per section. The percentage of vessel wall cells transduced was calculated.

Measurement of recombinant β-galactosidase activity and antigen. β-Galactosidase expression was evaluated in detergent extracts of excised arteries with two quantitative methods that measured (1) activity with use of MUG assays and (2) protein mass (antigen) with use of an enzyme-linked immunosorbent assay (ELISA) kit (5 Prime 3 Prime, Inc., Boulder, Colo.).

Arterial segments for MUG analysis were minced in lysis buffer that contained 0.2% Triton X-100 and Z-buffer (60 mmol/L sodium phosphate dibasic, 40 mmol/L sodium phosphate monobasic, 10 mmol/L potassium chloride, and 1 mmol/L magnesium sulfate, pH 7.0). Tissue debris was cleared by

centrifugation at 14,000*g* for 6 minutes. Fifty microliters of tissue extract was added to 650 μl of Z-buffer. The reaction was started by addition of 200 μl of 3 mmol/L MUG in Z-buffer. The reaction was monitored for 1 hour with use of a luminescence spectrometer (Perking-Elmer, Norwalk, Conn.), with excitation at 350 nm and emission at 450 nm. β-Galactosidase activity was determined on the basis of the linear portions of kinetic curves generated by use of purified *Escherichia coli* β-galactosidase (Sigma Chemical Co., St. Louis, Mo.), with specific activity ranging from 1.5 to 200 μU. Assay samples with light emission below that generated by the 1.5 μU standard were read as "undetectable."

The sensitivity of the MUG assay, along with inherent variation in the kinetic curves generated for assays completed on different days, led to variation in the individual standard curves generated for the activity assays. For this reason, variability of calculated activity occurred when animals assayed at different times were compared; for example, activity for the endogenous rat β-galactosidase in unoperated control arteries. In all cases, specimens from individual rats were assayed with a single standard. That is, although values for the right and left arteries varied according to which standard was applied because of the linearity properties of the standard, the right/left ratio remained constant. Therefore the ratio of recombinant protein activity in the transduced (right) artery versus the activity in the paired control (left) artery of each individual rat accurately reflected the changing levels of β-galactosidase activity over time or subsequent to varying vector concentrations. For this reason, this ratio (i.e., β-galactosidase activity of right artery/β-galactosidase activity of left artery) was calculated for each individual animal. The mean of these ratios was calculated for all animals is each experiment, and the mean of the ratios, rather than the absolute activity values, was used for comparing results between experiments.

Tissue extracts of artery segments for ELISA were prepared as described previously,[23] except that the adventitia was left intact on the specimens. ELISA assays gave a linear response to standard β-galactosidase concentrations between 100 to 2000 pg/mg of total protein. Total protein concentrations in vessel extracts were made with use of a standard protein determination kit (Pierce Chemical Co., Rockford, Ill.), with bovine serum albumin as a standard.

Evaluation of duration of recombinant β-galactosidase expression. Carotid arteries of 17 rats were used to assess β-galactosidase expression 24 hours after instillation of vector or control solutions, including (1) rAAV/Col (n = 10), (2) rAAV/CMV (n = 4), and (3) rAAV/pTRβ, the vector without a promoter, as a negative control (n = 3). One rAAV/Col rat underwent simultaneous instillation of normal saline solution into the contralateral (left) carotid artery to serve as a surgical control. Av1LacZ4, the adenoviral vector, was instilled in two rats. Av1LacZ4-instilled arteries were harvested at 72 hours, which was the interval used with this vector in previous studies that showed significant transgene expression.[15,23] Twenty-five additional rats were included in a time-course study with use of rAAV/Col as the vector. Arteries were harvested at varying intervals after in vivo gene transfer, as follows: 96 hours (n = 4), 1 week (n = 8), 2 weeks (n = 4), 1 month (n = 5), and 2 months (n = 4).

β-Galactosidase expression was evaluated in individual artery segments at each time interval with MUG assays and at 24 hours (n = 4) and 1 week (n = 3) with ELISA assays. DNA in situ PCR was used to assess the number and distribution of vessel wall cell nuclei that contained the lacZ gene at 24 hours, 1 week, and 2 months.

At intervals when vessels were evaluated by more than one type of assay, one vessel was divided into two segments and used for two assays, if possible, to minimize the number of animals required. (For example, when gene transfer was assessed with PCR and gene expression was measured with the MUG assay at 2 months after gene transfer). For this reason, the total sample size (n) for assays and stains exceeds the number of rats used in the study.

Evaluation of effect of viral titer on transduction and expression. To evaluate the effect of viral titer, eight additional rats were included in a dose-dependence study. Arteries were infused with 50 μl of a 1:1 dilution of rAAV/Col stock (final titer, 2.5×10^5 iu/ml; n = 4) or a 1:10 dilution (final titer, 5×10^4 iu/ml; n = 4) for comparison with arteries infused with undiluted rAAV/col stock (titer, 5×10^5 iu/ml; n = 6). The diluent was normal saline solution. β-Galactosidase expression was evaluated in individual segments with MUG assays.

Statistics. For each group of vessels or animals that received the same treatment in individual experiments, mean values were calculated from the values for individual vessels or animals constituting a group; for example, β-galactosidase activity levels, β-galactosidase antigen levels, and ratios of activity level in transduced versus control arteries. The Sign test was used to evaluate the significance of differences between transduced and control arteries; differences were considered to be significant at $p < 0.05$.

RESULTS

In vivo gene delivery and estimation of transduction frequency. Arteries were incubated with rAAV/Col (n = 6), rAAV/CMV (n = 4) or normal saline solution (n = 1) and harvested at 24 hours after gene delivery. For transduction frequency estimates, the number of PCR-stained cell nuclei per histologic section of whole vessel was counted for each vector, as well as the total number of cell nuclei per histologic section, and the percentage of positive cells was calculated. Results were expressed as the mean values ± SEM (n = 6 sections from two animals for each vector). With use of rAAV/Col, the total number of cell nuclei was 82 ± 6 per section, with 91% ± 1% of cells positive (range, 85.9% to 94%). With use of rAAV/CMV, the total number of cell nuclei was 84 ± 6 per section, with 89% ± 1% of cells positive (range, 84.6% to 92.2%). Thus in situ PCR amplification and staining of intimal and smooth muscle cell nuclei provided evidence for the presence of the foreign lacZ gene, compared to none in normal saline solution–instilled arteries and in unoperated control arteries. No evidence of significant intimal or medial cell loss or acute inflammatory cell infiltrates was noted after light microscopic analysis of sections of the infected vessel wall stained with hematoxylin and eosin (Fig. 1, *A* through *F*).

Level and duration of recombinant β-galactosidase expression. The level of β-galactosidase activity was assayed in individual vessel extracts at 24 hours after gene transfer from vessels instilled with rAAV/Col (n = 6), rAAV/CMV (n = 4), and rAAV/pTRβ (n = 3). Vessels instilled with Av1LacZ4 (n = 2) were harvested at 72 hours. Activity was expressed in mean microunits per milligram of total protein (Fig. 2). At 24 hours, the rAAV/Col vector mediated significant protein expression ($p = 0.015$), whereas the rAAV/CMV and rAAV/pTRβ vectors did not.

Although transduction with the Av1LacZ4 vector resulted in β-galactosidase activity more than 18-fold higher than in paired unoperated arteries the sample size was too small to allow statistical analysis. Because a high level of expression has been reported previously with use of this high-titer adenoviral vector,[23] our results suggest that the MUG assay accurately reflects β-galactosidase activity.

To assess the duration of expression, β-galactosidase activity was assayed in vessel extracts of arteries instilled with rAAV/Col at varying intervals that ranged from 96 hours to 2 months after gene transfer. The ratio of β-galactosidase activity in the transduced to the paired control vessel was calculated at each interval for each individual animal (data not shown), and the mean of these ratios was calculated and is indicated for each group (Fig. 2). Transgene expression decreased to a low level at 96 hours and remained detectable for 1 month after gene transfer ($p = 0.03$).

The quantity of β-galactosidase antigen was determined in artery extracts at 24 hours (n = 4) and 1 week (n = 3), and expressed in picograms per milligram of total protein (Fig. 3). Because the ELISA antigen assay is specific for *bacterial* β-galactosidase, any difference in antigen quantities must result from transgene expression. At 24 hours, the absolute value of the antigen levels in the transduced vessels exceeded those for control vessels. The values were not significantly different because of the small sample size, although a statistical trend was shown ($p = 0.06$).

DNA in situ PCR of representative segments at 1 week and 2 months after gene transfer showed that the percentage of cells that contained the transgene decreased over time. Two months after gene transfer, the nuclei of cells near the luminal surface contained lacZ (Fig. 1, *G* and *H*).

Effect of viral titer on transduction and expression. The level of β-galactosidase activity was assayed in vessel extracts of arteries instilled with rAAV/Col at titers that ranged from 5×10^4 iu/ml to 5×10^5 iu/ml (Fig. 3). Transgene expression decreased in a dose-dependent fashion in this range of vector concentrations, although β-galactosidase activity ratios subsequent to transduction with either diluted vector solution were essentially identical to those of the rAAV/pTRβ control vector. Thus transduction with use of any dilution of the vector stocks resulted in loss of detectable levels of expression.

DISCUSSION

In this study, we show in situ PCR evidence that, in an uninjured rat artery, AAV vectors driven by two distinct promoters can mediate in vivo gene transfer to a high percentage of vessel wall cells, despite an intact endothelial cell layer. In addition, gene transfer with use of rAAV/Col, a vector driven by the murine collagen α(I) promoter, is accompanied by expression of recombinant protein, detectable by activity and antigen assays of vessel extracts.

In contrast, gene transfer with the vector driven by the CMV immediate-early promoter and enhancer (rAAV/CMV) did not result in detectable levels of recombinant protein expression in the interval examined. Thus successful gene transfer, although demonstrable by in situ PCR, does not uniformly result in significant transcription and

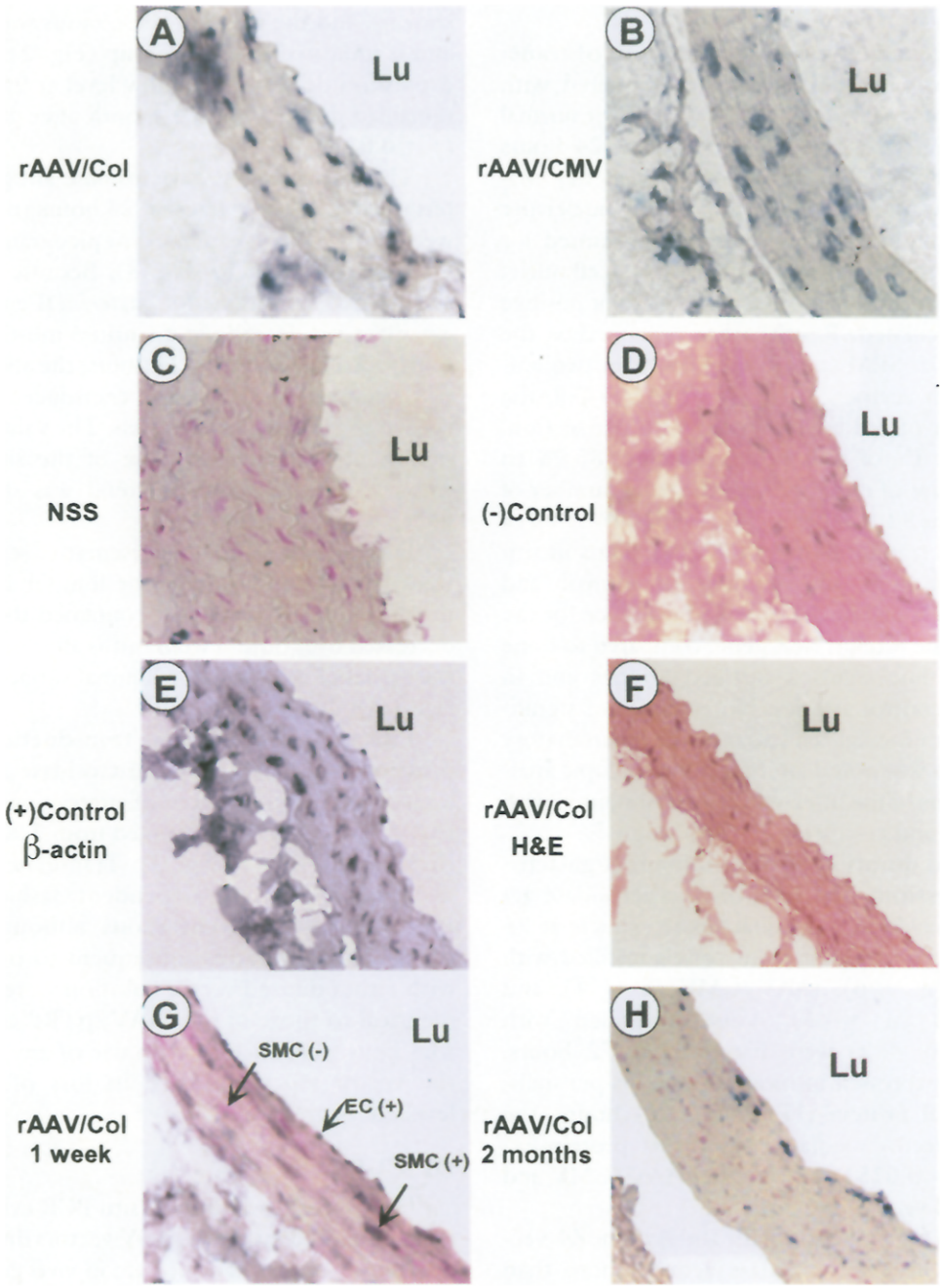

Fig. 1. Microscopic views of sections from rat carotid arteries (original magnification ×40). All vessels were stained by DNA in situ polymerase chain reaction (PCR) to detect the lacZ gene, except for panels **E** and **F**. *Lu* indicates the vessel lumen. *EC* and *SMC* indicate endothelial cell and smooth muscle cell, respectively. **A** through **D,** Show vessels harvested 24 hours after instillation of the solution indicated to the left of each photomicrograph. **A** and **B,** Show arteries transduced with individual recombinant adeno-associated virus (rAAV) vector solutions. *Dark blue staining* of cell nuclei provides evidence for the presence of the lacZ gene. **C,** Shows an artery instilled with normal saline solution. **D,** Shows an artery that has not been operated on. In panels **C** and **D,** no blue staining is present *(nuclear fast red stain)*. **E,** Shows a transduced artery stained by DNA in situ PCR to detect endogenous rat β-actin, to serve as a positive control. **F,** Shows an artery transduced with rAAV/Col and stained with hematoxylin and eosin stain. No significant cell loss or inflammatory infiltrates are present. **G** and **H,** Show DNA in situ PCR of arteries 1 week and 2 months after gene transfer with rAAV/Col, respectively. The percentage of cell nuclei that contain the lacZ gene decreases with time. At 2 months, lacZ is present in nuclei near the vessel lumen *(nuclear fast red stain)*.

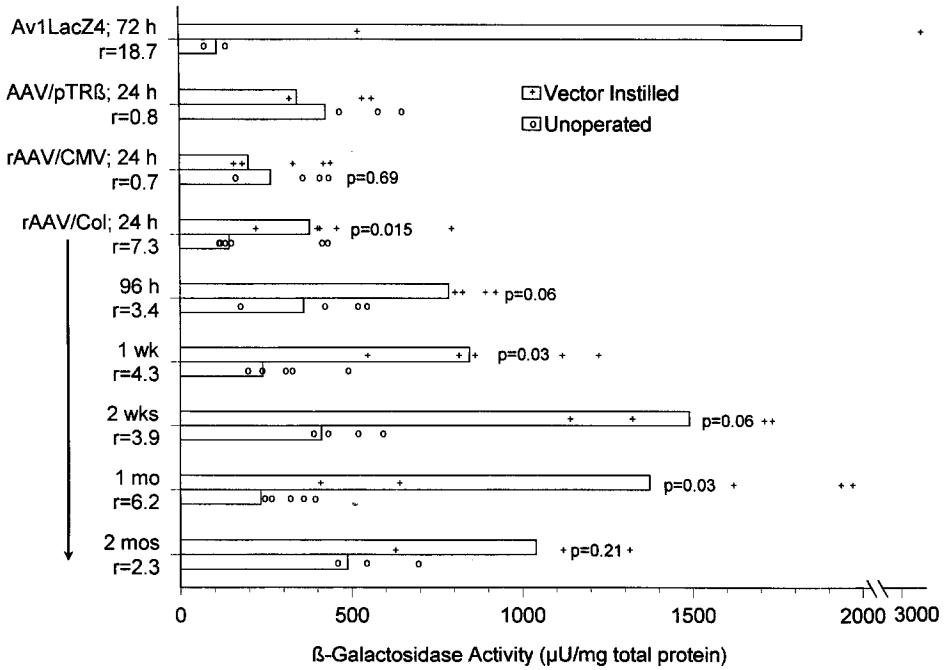

Fig. 2. Graph showing the level of β-galactosidase expression at the interval indicated after in vivo gene transfer. The vector instilled is indicated to the left of each pair of bars. *Bars with plus signs (+)* represent arteries instilled with a vector solution; *bars with circles (○)* represent paired unoperated control arteries. Activity is expressed as microunits per milligram of total protein in the tissue extract. *Bars* indicate the mean β-galactosidase activity in each group. Each *data point* represents a single vessel. For each group, *r* represents the mean of the ratios that compare activity in the instilled versus the paired unoperated artery for each individual animal in that group (see text). Where *p* values are absent, the sample size (n) was too small to allow statistical analysis.

translation of the foreign gene, as emphasized by Halbert et al.[25]

Although the level of protein expression obtained with rAAV vectors was 1000-fold less than the level reported with adenoviral vectors (i.e., picograms versus nanograms per milligrams of total protein), the titers of rAAV vectors used in this study were 100,000-fold lower (10^5 iu/ml) than the titers of the adenoviral vectors (10^{10} pfu/ml) used in the previous experiments.[15,23] Transduction with more concentrated rAAV stocks may result in translation of higher levels of protein.

Expression of recombinant protein continues at low levels for 1 month after rAAV-mediated gene transfer. The mean ratio of activity in the transduced versus control vessels is highest (more than sevenfold) at 24 hours, decreases by 96 hours (3.4-fold), and remains essentially constant at a low level for 1 month. In comparison, expression after adenoviral transduction in an injured artery model persisted at high levels for 7 days, then declined to a low level, and remained detectable for 42 days.[23] Whether or not long-term expression from recombinant AAV vectors is related to stable integration into the cell genome remains to be determined.

In situ PCR 1 week and 2 months after transduction show that, over the course of the study, the transgene persists in endothelial cells and in the smooth muscle cells nearest to the vessel lumen, whereas it diminishes in the smooth muscles cells farther from the lumen. We hypothesize that the cells nearest to the arterial lumen may initially receive higher copy numbers of the transgene as a result of the luminal delivery of the vectors. The quiescent nature of endothelial cells, in contrast to the higher turnover rate of smooth muscle cells, may also contribute to the observed persistence of the transgene in the endothelium for up to 2 months.

Arterial transduction with high-titer adenoviral agents results in significant toxicity.[15,16] Although we did not assess vessels with specific chemical stains for inflammatory cells, microscopic evaluation of typical sections did not show evidence of gross inflammation or cell loss. Whether high-titer rAAV vectors cause tissue toxicity in vivo is unknown.

Transduction of intact arteries in vivo with use of adenoviral vectors results in endothelium-specific gene transfer,[26,27] probably because of the impermeability of the endothelium or subendothelium to particles the size of adenovirus (diameter ≈80

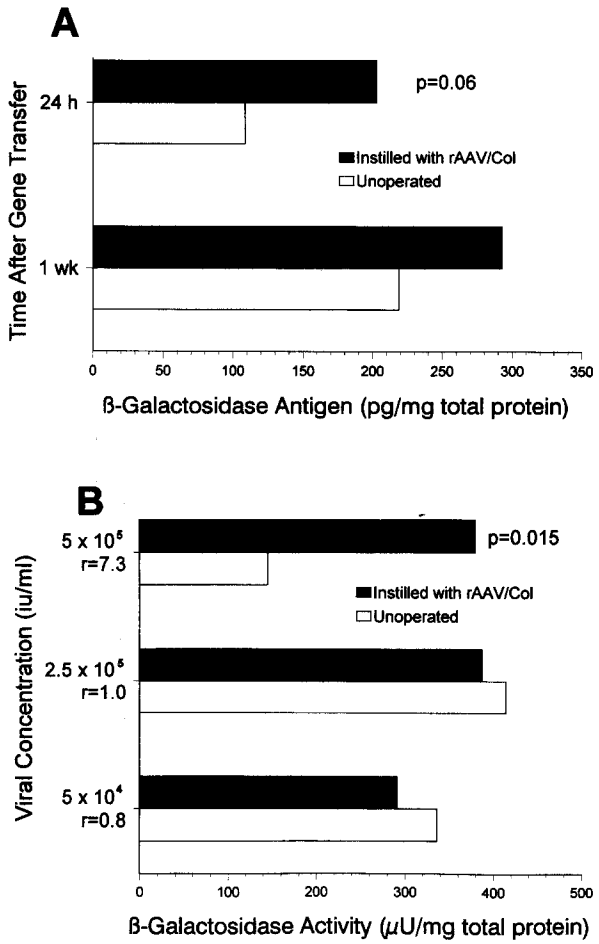

Fig. 3. Graphs showing β-galactosidase antigen at time intervals of 24 hours and 1 week (**A**) and β-galactosidase activity at varying virus concentrations (**B**). *Solid bars* represent arteries incubated with rAAV/Col vector solution. *Open bars* represent paired unoperated control arteries. Expression is expressed as picograms (**A**) or microunits (**B**) per milligram of total protein in the tissue extract. *Bars* indicate the mean β-galactosidase expression in each group. Each *data point* represents a single vessel. For each group in graph **B**, *r* represents the mean of the ratios that compare activity in the instilled versus the paired unoperated artery for each individual animal in that group (see text). Where *p* values are absent, the sample size (n) was too small to allow statistical analysis. *iu,* Infectious units.

nm).[28,29] In contrast, the diameter of AAV (only ≈22 nm) may allow rAAV-based vectors to penetrate the intact arterial wall. The mode of uptake of wtAAV has not been characterized, and the cellular receptor has not been identified.[8] Unique features of AAV infection may explain its apparent ability to transfect the media of an intact vessel after luminal delivery, as suggested by this study. In addition, the intraluminal pressures used in this study for vector delivery (150 to 190 mm Hg) are similar to those recommended by Nabel et al.[30] for optimal in vivo transfer throughout an intact vessel wall with use of DNA-liposome complexes, which are significantly larger (diameter ≈250 nm)[29] than rAAV vectors.

Current methods for gene transfer targeting medial cells are inefficient.[28] Gene transfer throughout an uninjured vessel wall would be useful for studying the effect of specific genes in a normal artery. Our study suggests that AAV vectors may allow direct gene transfer targeted to the medial smooth muscle cells, without requiring mechanical disruption of the intima. However, levels of gene expression were very low. Recent advances in methods now allow production of large quantities of high-titer rAAV vectors.[12,31] Transduction of arteries with high-titer rAAV stocks is likely to result in higher levels of expression, making possible the use of histochemical stains to detect recom-

binant protein, which will facilitate in vivo investigations.

Perhaps because AAV is not considered to be a human pathogen, basic questions regarding the biology of AAV-based vectors remain unanswered. Issues that could be addressed in vivo include (1) vector integration versus persistence as an episome, (2) site specificity of integration, and (3) toxicity of high-titer vectors. The potential for targeted in vivo transduction of medial cells in intact arteries could be examined, with the ultimate goal of transferring a gene encoding a therapeutic protein.

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